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Increased Antibody Affinity and Specificity by Codon-Based Mutagenesis

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The specificity and affinity of antibody combining sites towards antigen is governed primarily by the amino acid sequence within the six hypervariable regions of the antibody variable (V) domain, three of which are contributed by the heavy chain V region (V_H) and three by the light chain V region (V_L). Affinity maturation, the process whereby antibody affinity increases with time during an immune response, relies on V gene somatic mutation and antigen affinity-driven selection. Rearranged germline antibody genes accumulate mutations at a rate estimated to be between 10^{-4} to 10^{-3} per base pair per cell division and these mutations are primarily localized to the hypervariable or complementarity-determining regions CDR1, CDR2, and CDR3 of both V_H and V_L genes and their flanking sequences [1–3].

Recent advances in the molecular cloning and expression of antibody gene fragments in bacteria such as *E. coli* have created opportunities for engineering the antibody combining site with the goal of improving affinity towards antigen or redirecting binding specificity [4,5]. This is particularly desirable for therapeutically promising human antibodies that suffer from insufficient affinity toward target antigen or unwanted cross-reactivity with nontarget tissue. Several unique strategies have been successfully used to genetically alter antibody binding properties. Marks *et al.* [6] have constructed a high affinity derivative of a single chain human anti-z-phenyloxazol-5-one antibody fragment by a process termed chain shuffling. Briefly, this procedure involves the sequential reassortment of V_H and V_L genes with repertoires of V region genes to generate a pool of novel binding specificities. Enrichment for antibody fragments on immobilized antigen yielded a number of higher affinity antibodies almost all of which were subsequently determined to have resulted from a slower off rate.

Error prone polymerase chain reaction (PCR) has been used by Gram and colleagues to introduce mutations into an anti-progesterone single chain antibody fragment expressed in a filamentous phage display system [7]. This technique allowed the selection of antibody fragments with up to 30-fold higher affinity towards the hapten antigen relative to the parent antibody molecule.

We have recently described a method of oligonucleotide-directed mutagenesis in an M13 filamentous phage system that introduces mutations within the antibody combining site by replacing entire codons rather than individual nucleotides [8]. Mimicking affinity matura-

tion *in vitro* requires the efficient substitution of amino acids within target regions without causing deleterious changes in the combining site architecture. Codon-based mutagenesis accomplishes this by allowing the synthesis of oligonucleotides with any degree of bias towards parental sequence. Codon-based mutagenesis has certain advantages compared to single nucleotide mutagenesis, as occurs during *in vivo* hypermutation or other *in vitro* mutagenesis procedures [9,10]. *In vivo* and conventional mutagenesis procedures generally introduce single nucleotide changes. However, the introduction of some potentially beneficial amino acids may require the replacement of all three nucleotides within the wild type codon. It is unlikely that conventional *in vitro* mutagenesis techniques could produce these changes at mutation rates (*i.e.* the number of mutations per base required to change three contiguous nucleotides within a target sequence of defined length) that would not effect additional nucleotide residues. In contrast, codon-based mutagenesis can efficiently effect amino acid substitutions even at codons where the target and the mutagenic oligonucleotide sequences are dissimilar. In addition, oligonucleotides can be designed to preserve key amino acid residues that might be involved in maintaining the conformation of the CDR loops [11].

The first step towards the expression and optimization of antibody fragments in *E. coli* is to clone the genes of interest. The target antibody gene sequences can be constructed from combinatorial antibody libraries or by directly recombining specific heavy and light chain fragments cloned into an antibody expression system such as the M13IX31 and M13IX12 vectors recently described [12,13]. Upon determining the target DNA sequence, codon-based oligonucleotides are synthesized and used to mutagenize single-stranded antibody V-region DNA prepared from M13 phage containing the cloned antibody F(ab) gene fragments. Following mutagenesis variant F(ab)s expressing the desired antigen binding properties are isolated and characterized. A diagram illustrating the DNA sequence (and corresponding amino acids) of a random sampling of a library generated by codon-based mutagenesis and the resultant M13 clones identified by phenotypic selection is shown in Figure 1. The oligonucleotides used for mutagenizing the target CDR were synthesized to maintain a 50% parental sequence bias for each codon within the target CDR. This is reflected by the presence of a parental amino acid residue at each position within the target CDR in half of the sequences sampled. In this particular example, the library is screened for variant F(ab)s expressing higher affinity to antigen. DNA sequencing of the selected variant F(ab)s reveals the position and type of amino acid(s) that confer the selected phenotype. In the example shown in Figure 1, the selected clones all have a small aliphatic residue at position 31 replacing the parental glutamic acid.

Screening strategies are highly dependent upon the nature of the antigen of interest. As shown in Figure 2, soluble, well characterized antigens can be labeled and used to probe variant F(ab) libraries in a filter lift assay. Various conditions and strategies can be used to identify novel F(ab) fragments presenting the desired phenotypes. For example, we recently applied codon-based mutagenesis in combination with filter lift assays to identify improved affinity F(ab)s by modifying the wash conditions following incubation with labeled antigen. Chimeric L6 F(ab), a monoclonal antibody that recognizes a tumor-associated cell surface antigen expressed by many human carcinomas served as the model antibody [14–18]. An anti-idiotype antibody that binds near the L6 antigen binding site was used to represent the target antigen [19]. The screening strategy, outlined in Figure 2, succeeded in identifying 5 related clones from an initial population of approximately 5000 clones that upon further evaluation were found to have affinities approaching 10–15-fold higher than the parent L6

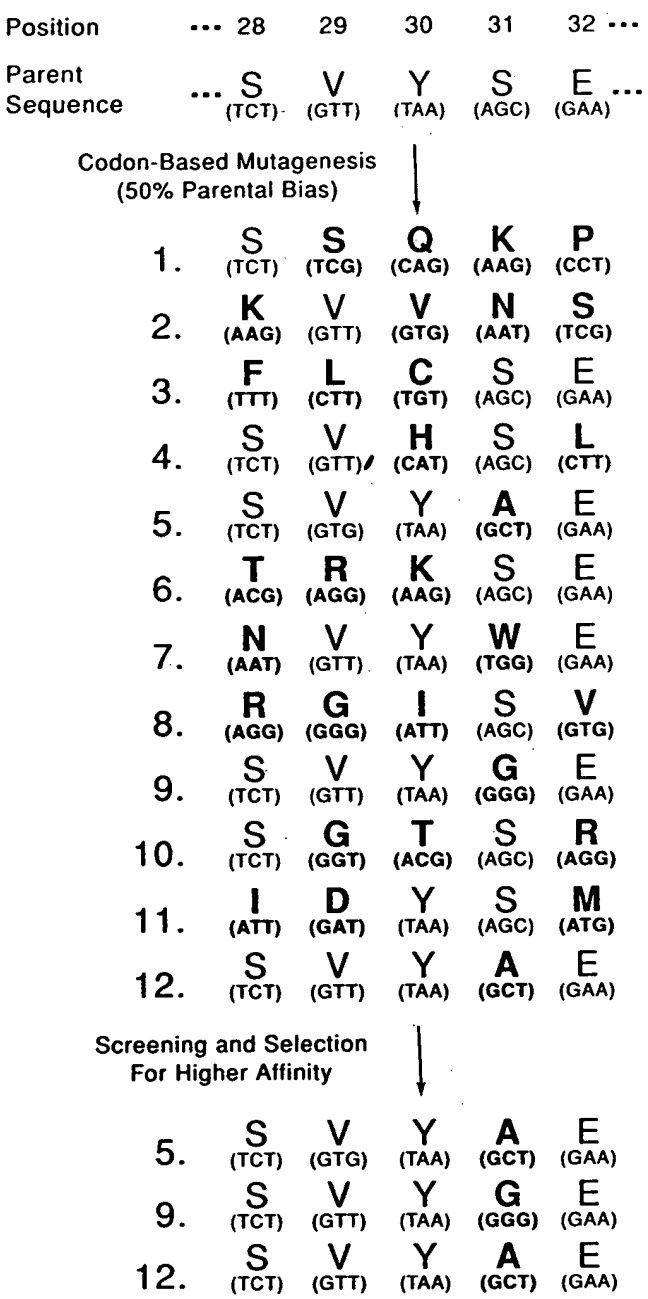

FIGURE 1 An example of mutant antibody sequences generated by codon-based mutagenesis. The position, codon sequence, and corresponding amino acid sequence of five target residues within the parental CDR is indicated. Clones 1–12 represent a random sampling of the library. Changes resulting from the mutagenesis procedure are indicated by bold type. Clones 5, 9, and 12 have been identified from the library by stringent affinity-based interaction with antigen. Clone 5 has a GTG coding for Val rather than the parental GTT indicating that Val in this position is required. Single letter symbol: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; Q, Gin; E, Glu; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; and V, Val.

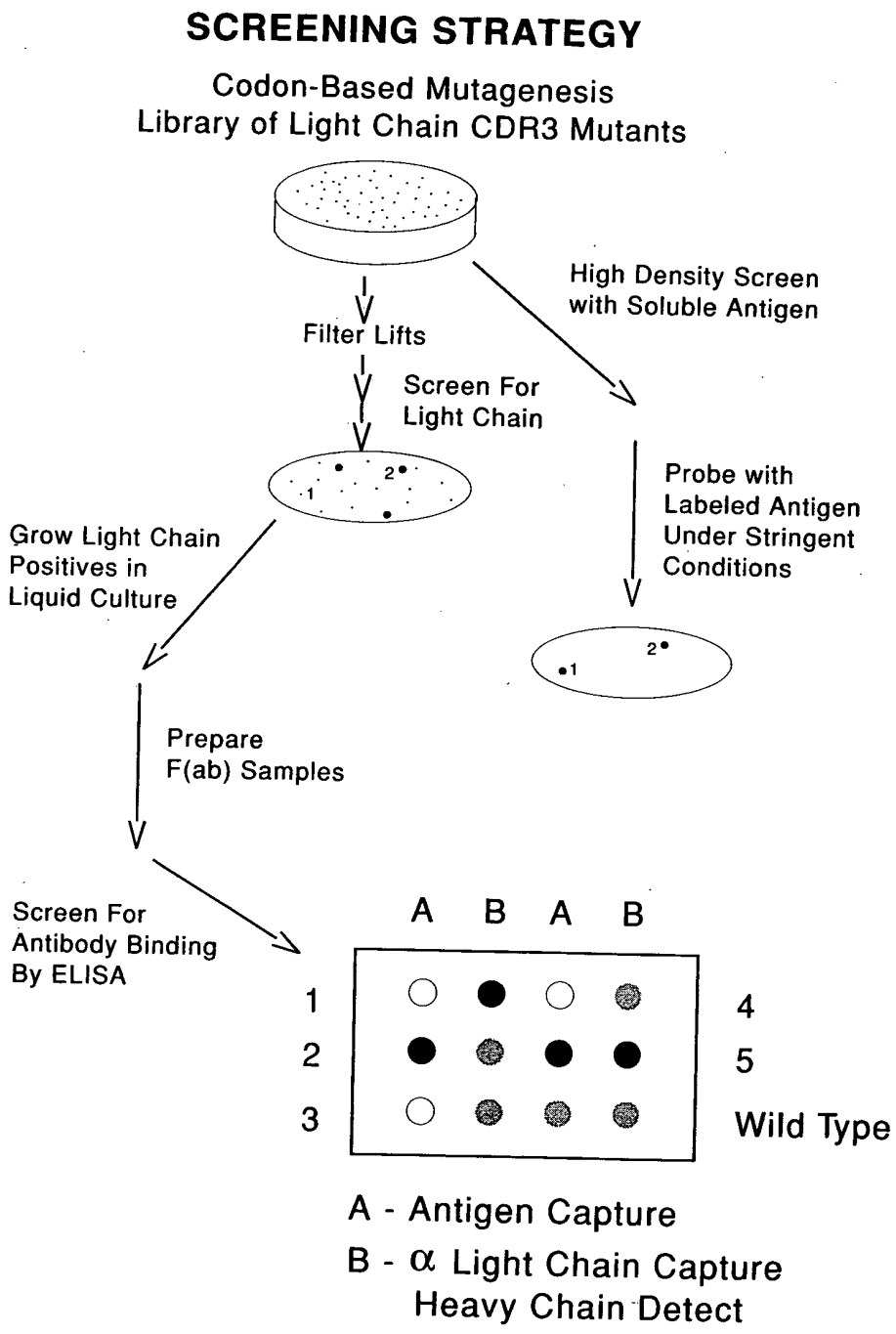

Fig 2

FIGURE 2 Strategies for screening variant F(ab) libraries. Soluble, well characterized antigens can be used to screen F(ab)s by filter lift assay. Complex or insoluble antigens can be used to screen for modified antibody fragments by ELISA. In the schematic diagram of the ELISA assay black=strong signal, grey=medium signal, and white=weak signal.

F(ab) as determined by surface plasmon resonance (BIAcore™, Pharmacia) (Cosand, W., Glaser, S., Huse, W., and Yelton, D., manuscript in preparation) [20].

It is important to consider the effect of F(ab) expression levels when screening for higher affinity antibodies. For monovalent interactions the affinity of an antibody (Ab) for antigen (Ag) is equivalent to the equilibrium constant K. The formation of antibody-antigen complexes (AgAb) can then be described by the equation K=[AgAb]/[Ag][Ab]. If [Ab] increases, mass action drives the formation of AgAb complex. In practice, for a given concentration of Ag, this will be observed as a darker signal compared to parental F(ab) in the filter lift probed with antigen, easily misinterpreted as a "higher affinity" clone (see clone 2 on the filter lift in Fig. 2). Therefore, when searching for higher affinity antibody fragments it is recommended to prepare a replica filter lift and probe this lift with an anti-F(ab) antibody to evaluate the contribution of F(ab) expression towards the overall signal detected with labeled antigen. In this example, clone 1 is clearly the candidate of choice for further evaluation.

Insoluble or incompletely characterized antigens such as tumor-associated cell surface antigens can be used in screening variant F(ab) libraries by conventional enzyme-linked immunosorbant assay (ELISA) (Fig. 2). M13 phage plaques that test positive for presence of intact V_H or V_L (a diagnostic for the successful incorporation of a mutagenic oligonucleotide—see Ref. 8) are selected and grown in liquid culture and soluble F(ab) fragments prepared as described [13]. These F(ab) preparations are then used in standard ELISA assays and candidate clones identified and selected for further characterization. In the example shown in Figure 2, clone 2 is a candidate because it has high antigen binding yet secretes normal levels of F(ab) compared to wild type. In spite of the relatively low throughput compared to the filter lift assay, we have screened two independent chimeric L6 codon-based mutagenized libraries, each with a different mutagenized CDR against tumor cells using ELISA assay and have successfully identified higher affinity clones (Stinchcombe, T., Roth, K., Yelton, D., and Huse, W., unpublished results).

An adaptation of the two strategies outlined in Figure 2 has also been used to screen for variant L6 antibodies with altered specificity. Several anti-idiotype (anti-id) antibodies previously raised against the L6 antibody were used to represent target and nontarget antigens [19]. The goal was to eliminate binding activity towards a model cross-reactive anti-id while preserving L6 reactivity towards one or more of the remaining anti-ids [8]. A set of replica filter lifts were prepared from a portion of an L6 library containing two mutagenized CDRs. One lift was screened against the desired target anti-id and the other was screened against the cross-reactive nontarget anti-id. A comparative analysis of the two filters allowed us to readily identify variant F(ab)s that preserved wild type binding to target anti-id, yet completely lost binding activity towards the cross-reactive anti-id.

Codon-based mutagenesis has been used to optimize both affinity and specificity of the L6 antibody combining site towards model antigens in a high throughput filter lift assay. The development of alternative screening procedures, such as ELISA, have subsequently allowed the identification of higher affinity L6 clones to tumor antigen. In both cases, those antibodies with increased affinity and specificity have been identified from populations at frequencies equal to or less than 1 in 1000 mutants. Developing a functional assay that either quantitatively or qualitatively reflects the intrinsic binding property of the molecule of interest is central to a successful screen. Because binding constants are by definition thermodynamic quantities which represent an average of the number of moles of ligand bound per mole of antibody, a signal produced in a predictive functional assay consists of an average of many molecular binding events. Plaque lift and ELISA assays inherently display

these characteristics. Whereas affinity chromatography techniques tends to be attractive for screening large numbers of variant antibody fragments when displayed on the phage surface, they are sensitive to individual molecular events and therefore may not be predictive of thermodynamic properties such as affinity constants. Since populations of less than one thousand, or in some cases a few hundred mutants, actually possess the desirable alterations, it is more feasible to screen these using plaque lift and ELISA assays.

Although these studies yielded the desired binding phenotypes with mutations limited to only one or two CDRs, it is foreseeable that there will be examples where optimization of an antibody will require multiple mutations distributed among several CDRs. Three possible strategies on how one might approach a more complicated antibody combining site optimization are presented in Figure 3. For sake of discussion, the goal will be to identify higher affinity antibody fragments. Figure 3A diagrams a strategy where three individual codon-based mutagenized CDR libraries are constructed, one unique mutagenized CDR per library (*i.e.* CDR1 light chain, CDR2 light chain, etc.). Each library is then screened for higher affinity clones. The DNA sequences of the resulting higher affinity clones are determined and oligonucleotides encoding these new sequences synthesized. These oligonucleotides are then used to construct pairwise combinations to create a second generation of variant F(ab)s. Because this is conducted in the M13 phage system all of the combinations can be quickly and efficiently constructed by site-directed mutagenesis. The second generation of F(ab)s are subjected to the same screening procedure and the process repeated as necessary. Though strategy 3A assumes that each of the amino acid contact sites comprising the antibody-antigen interface are functionally independent, there is precedence that the combined effect will produce antibodies possessing overall superior binding properties [21]. In addition, a significant advantage of constructing the combination libraries is that is restricts the size of each library to manageable numbers.

A second strategy for antibody optimization is diagrammed in Figure 3B. In this strategy the mutagenesis procedure is easily extended to include any number of CDRs. This is done by first constructing a library consisting of two or three mutagenized CDRs on the heavy chain and a separate library consisting of two or three mutagenized CDRs on the light chain. Recombination of the two libraries results in a large combinatorial library in which any desired number of CDRs can be modified. The major obstacle with this approach is developing a reliable screening method for such a large library.

In contrast to strategy 3A, the strategy presented in Figure 3C assumes that the higher affinity clone is derived from the stepwise accumulation of mutations. A single CDR library (*i.e.* CDR1 light chain) is screened and one or more high affinity clones identified and their DNA sequence determined. The novel amino acid(s) from light chain CDR1 are then introduced into the antibody template sequence and codon-based mutagenesis used to construct a new library in a completely different target CDR, such as CDR2. Reiterative rounds of mutagenesis and screening consequently result in a higher affinity antibody built upon the existing genetically modified background. We are currently experimenting with all three strategies in our laboratory.

In conjunction with the M13IX31 and M13IX12 vector system, codon-based mutagenesis provides a general approach for engineering clinically and scientifically useful monoclonal antibody fragments. These fragments can be created to remove unwanted cross-reactivity, enhance affinity toward the target antigen, modify effector activity and introduce reactive residues for conjugating imaging or therapeutic agents. In the past, the *in vivo* diagnostic and therapeutic application of antibodies has been limited mainly by their murine or non-human origin. Since the advent of chimeric, humanized, and even entirely human

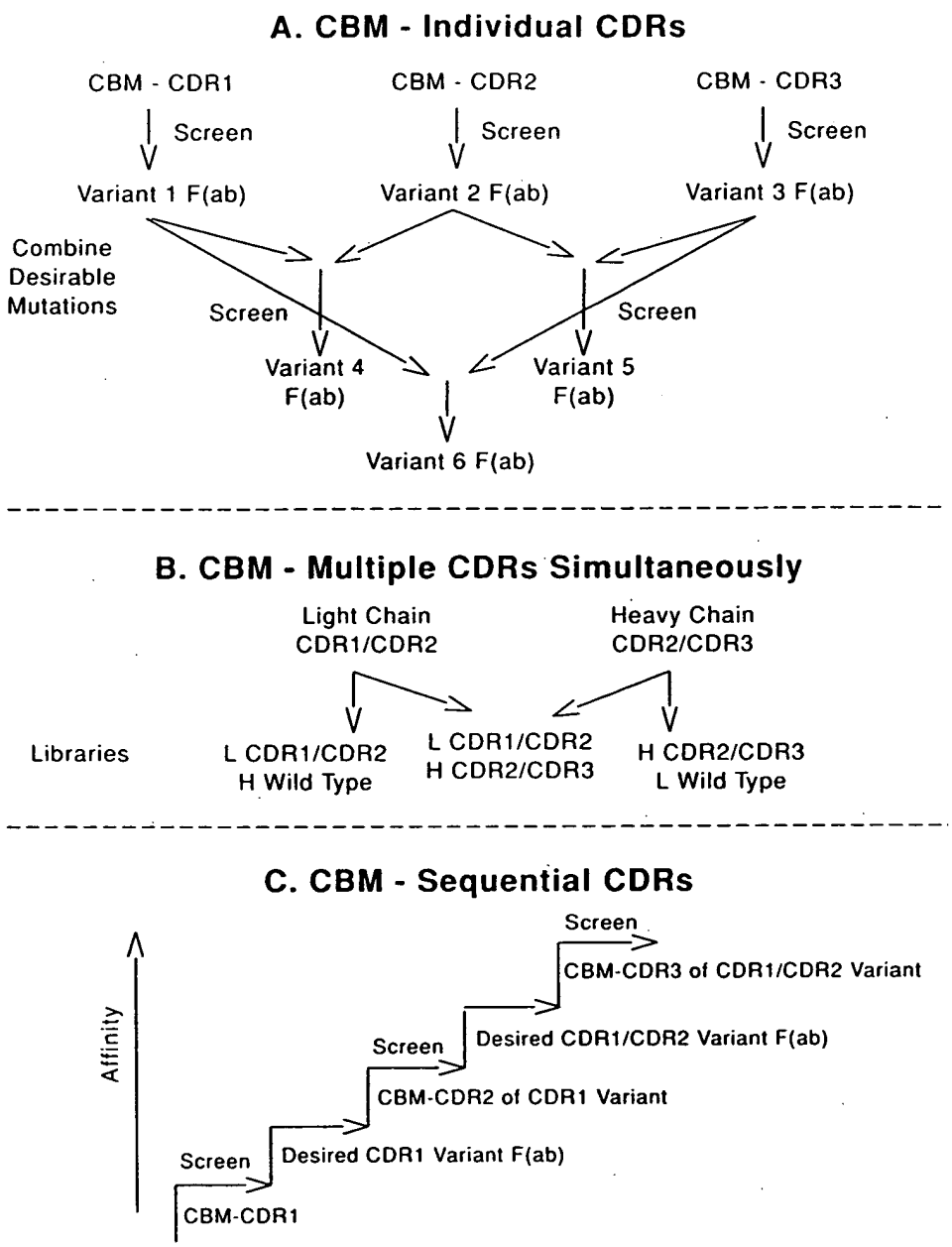

FIGURE 3 Various applications of codon-based mutagenesis (CBM) for producing complex CDR mutant libraries and optimizing antibody binding properties.

antibodies by *in vitro* methods, the primary factor affecting antibody quality is the lack of high specificity and affinity. Already the progress observed with the development of efficient antibody engineering procedures is encouraging and promises to circumvent these problems and lead to the immunotherapeutic treatment of human diseases [22–24]. The clinically beneficial application of monoclonal antibodies for treating cancer, infectious diseases, autoimmune diseases, and inflammation relies upon a multitude of complex factors. Among these are the pharmacokinetic properties, penetration of the drug into the target tissue, the mechanism of cell killing, and the mechanism of neutralization of foreign substances by antibody [24,25]. For those antibodies that can not recruit immunological effector function, therapeutic efficacy may require derivatizing the candidate antibody to a drug, toxin, or radionuclide. Monoclonal antibodies can also be used to deliver enzymes to target cells in order to convert drug precursors into a pharmacologically active form. Many of these issues can hopefully be addressed by capitalizing on the antibody engineering techniques now available.

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References

1. Berek, C. and Milstein, C. Mutation drift and repertoire shift in the maturation of the immune response. *Immunol. Rev.* **96**, 23, 1987.
2. Allen, D., Cumano, A., Dildrop, R., Kocks, C., Rajewsky, K., Rajewsky, N., Roes, J. Sablitzky, F., and Siekevitz, M. Timing, genetic requirements and functional consequences of somatic hypermutation during B-cell development. *Immunol. Rev.* **96**, 5, 1987.
3. Berek, C. and Milstein, C. The dynamic nature of the antibody repertoire. *Immunol. Rev.* **105**, 5, 1988.
4. Plückthun, A. Antibodies from *Escherichia coli*. *Nature* **237**, 497, 1990.
5. Winter, G. and Milstein, C. Man-made antibodies. *Nature* **349**, 293, 1991.
6. Marks, J. D., Griffiths, A. D., Malmqvist, M., Clackson, T. P., Bye, J. M., and Winter, G. Bypassing immunization: building high affinity human antibodies by chain shuffling. *Bio/technology* **10**, 779, 1992.
7. Gram, H., Marconi, L.-A., Barbas, C. F., Collet, T. A., Lerner, R. A., and Kang, A. S. *In vitro* selection and affinity maturation of antibodies from a naive combinatorial immunoglobulin library. *Proc. Natl. Acad. Sci. USA* **89**, 3576, 1992.
8. Glaser, S. M., Yelton, D. E., and Huse, W. D. Antibody engineering by codon-based mutagenesis in a filamentous phage vector system. *J. Immunol.* **149**, 3903, 1992.
9. Reidhaar-Olson, J. F. and Sauer, R. T. Combinatorial cassette mutagenesis as a probe of the informational content of protein sequences. *Science* **241**, 53, 1988.
10. Derbyshire, K. M., Salvo, J. J., and Grindley, N. D. F. A simple and efficient procedure for saturation mutagenesis using mixed oligodeoxynucleotides. *Gene* **46**, 145, 1986.
11. Chothia, C., Lesk, A. M., Tramontano, A., Levitt, M., Smith-Gill, S. J., Air, G., Sheriff, S., Padlan, E. A., Davies, D., Tulip, W. R., Colman, P. M., Spinelli, S., Alzari, P. M., and Poljak, R. J. Conformation of immunoglobulin hypervariable regions. *Nature* **342**, 877, 1989.
12. Huse, W. D. Combinatorial antibody expression libraries in fliamentous phase. In: *Antibody Engineering: A Practical Guide*, C. A. K. Borrebaeck, Ed., New York: W. H. Freeman and Company, 1991, 103–120.
13. Huse, W. D., Stinchcombe, T. J., Glaser, S. M., Starr, L., MacLean, M., Hellström, K. E., Hellström, I., and Yelton, D. E. Application of a filamentous phage pVIII fusion protein system suitable for efficient production, screening and mutagenesis of F(ab) antibody fragments. *J. Immunol.* **149**, 3914, 1992.
14. Hellström, I., Horne, D., Linsley, P., Brown, J. P., Brankovan, V., and Hellström, K. E. Monoclonal mouse antibodies raised against human lung carcinoma. *Cancer Res.* **46**, 3917, 1986.
15. Hellström, I., Beaumier, P. L., and Hellström, K. E. Antitumor effects of L6, an IgG2a antibody that reacts with most human carcinomas. *Proc. Natl. Acad. Sci. USA* **83**, 7059, 1986.

16. Liu, A. Y., Robinson, R. R., Hellström, K. E., Murray, E. D., Jr., Chang, C. P., and Hellström, I. Chimeric mouse-human IgG1 antibody that can mediate lysis of cancer cells. *Proc. Natl. Acad. Sci. USA* **84**, 3439, 1987.
17. Fell, H. P., Gayle, M. A., Yelton, D., Lipsich, L., Schieven, G. L., Marken, J. S., Aruffo, A., Hellström, K. E., Hellström, I., and Bajorath, J. Chimeric L6 anti-tumor antibody. *J. Biol. Chem.* **267**, 15552, 1992.
18. Goodman, G. E., Hellström, I., Brodzinsky, L., Nicaise, C., Kulander, B., Hummel, D., and Hellström, K. E. Phase I trial of murine monoclonal antibody L6 in breast, colon, ovarian, and lung cancer. *J. Clin. Oncol.* **8**, 1083, 1990.
19. Hellström, K. E., Yelton, D. E., Fell, H. P., Beaton, D., Gayle, M., MacLean, M., Kahn, M., and Hellström, I. Epitope mapping and use of anti-idiotype antibodies to the L6 monoclonal anticarcinoma antibody. *Cancer Res.* **50**, 2449, 1990.
20. Jönsson, U., Fägerstam, L., Ivarsson, B., Johnsson, B., Karlsson, R., Lundh, K., Löfås, S., Persson, B., Roos, H., Rönnberg, I., Sjölander, S., Stenberg, E., Stählberg, R., Urbaniczky, C., Östlin, H., and Malmqvist, M. Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology. *Bio Techniques* **11**, 620, 1991.
21. Sharon, J. Structural correlates of high antibody affinity: Three engineered amino acid substitutions can increase the affinity of an anti-*p*-azophenylarsonate antibody 200-fold. *Proc. Natl. Acad. Sci. USA* **87**, 4814, 1990.
22. Morrison, S. L., Wims, L., Wallick, S., Tan, L., and Oi, V. T. Genetically engineered antibody molecules and their application. In: *Biological Approaches to the Controlled Delivery of Drugs*, R. L. Juliano, Ed., *Ann. N. Y. Acad. Sci.* **507**, p. 187, 1987.
23. Mayforth, R. D. and Quintáns, J. Designer and catalytic antibodies. *N. Engl. J. Med.* **323**, 173, 1990.
24. Rodwell, J. D. Engineering monoclonal antibodies. *Nature* **342**, 99, 1989.
25. Waldmann, T. A. Monoclonal antibodies in diagnosis and therapy. *Science* **252**, 1657, 1991.